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Costamere Protein Expression and Tissue Composition of Rotator Cuff Muscle After Tendon Release in Sheep

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ABSTRACT: Previous studies suggested that degradation of contractile tissue requires cleavage of the costamere, a structural protein complex that holds sarcomeres in place. This study examined if costamere turnover is affected by a rotator cuff tear in a previously established ovine model. We found the activity of focal adhesion kinase (FAK), a main regulator of costamere turnover, was unchanged at 2 weeks but decreased by 27% 16 weeks after surgical release of the *infraspinatus* tendon. This was accompanied by cleavage of the costamere protein talin into a 190 kDa fragment while full length talin remained unchanged. At 2 weeks after tendon release, muscle volume decreased by 17 cm³ from an initial 185 cm³, the fatty tissue volume was halved, and the contractile tissue volume remained unchanged. After 16 weeks, the muscle volume decreased by 36 cm³, contractile tissue was quantitatively lost, and the fat content increased by 184%. Nandrolone administration mitigated the loss of contractile tissue by 26% and prevented fat accumulation, alterations in FAK activity, and talin cleavage. Taken together, these findings imply that muscle remodeling after tendon release occurs in two stages. The early decrease of muscle volume is associated with reduction of fat; while, the second stage is characterized by substantial loss of contractile tissue accompanied by massive fat accumulation. Regulation of costamere turnover is associated with the loss of contractile tissue and seems to be impacted by nandrolone treatment. Clinically, the costamere may represent a potential intervention target to mitigate muscle loss after a rotator cuff tear. © 2017 The Authors. *Journal of Orthopaedic Research* published by Wiley Periodicals, Inc. on behalf of the Orthopaedic Research Society. J Orthop Res

Keywords: rotator cuff tear; fatty infiltration; atrophy; costamere; tenotomy

Tears of rotator cuff (RC) tendons are frequent and affect a large portion of the elderly population.^{1,2} These tears lead to retraction, fat accumulation, and atrophy of the musculotendinous unit.³ To reestablish normal shoulder function, RC tendon tears are often surgically repaired, and the degree of muscle atrophy and fat accumulation correlates with the probability of repair failure.^{4,5} Until recently, these tendon tear-induced changes were considered irreversible.^{3,6} Thus, it is important to characterize these alterations to find solutions for their prevention and treatment and allow a successful recovery from a RC tear.

Tendon release induces a broad range of adaptations in the muscle, with reduced muscle length most frequently described.⁷ Regulation of muscle length occurs by adding (longitudinal hypertrophy) or removing (longitudinal atrophy) contractile material (i.e., sarcomeres) at the ends of myofibrils⁸ and it has been shown that tendon release leads to a reduced number of sarcomeres in series.⁹ Sarcomeres are tightly incorporated in a myofibril and cannot simply be removed and/or

degraded,¹⁰ as they are held in place by the costamere, a structural protein complex that links the Z-line to the extracellular matrix.¹¹ Therefore, longitudinal atrophy first requires disruption of the structural anchors (i.e., the costameres) by specific Ca²⁺-activated proteases¹² to release the respective sarcomeres. In fact, Z-line streaming is observed after tendon release in the rat¹³ and after spontaneous tendon tears in various human muscles.¹⁴

The main regulator of costamere turnover is focal adhesion kinase (FAK), an integrin-associated phosphotransfer kinase that is activated by phosphorylation on its tyrosine 397 site (pY397). It is mechanosensitive and reacts to altered loading of the muscle.¹⁵ Changes in protein concentrations of FAK and FAK-pY397 after reduced muscle loading, for example, unloading of human *m. vastus lateralis*¹⁶ or tendon release of the rat *m. soleus*¹⁷ are well described. However, it is currently unknown whether FAK, FAK-pY397, and concomitant alterations in costamere turnover are affected by RC tears.

Apart from early surgical repair, another treatment option, which has been described in sheep⁶ and rabbits,¹⁸ is weekly administration of the anabolic steroid nandrolone decanoate, which prevented fat accumulation when treatment was started with the onset of the tear. To our knowledge, the action of anabolic steroids on the costamere is not yet described after tendon release. Nevertheless, nandrolone decreased cytosolic Ca²⁺ in the mouse *m. soleus* after hindlimb unloading.¹⁹ This may result in lower Ca²⁺-activated protease activity and thereby decreased costamere cleavage, which potentially explains protection from muscle loss.^{10,12,20} In the ovine model of RC tear, the reduction of total muscle

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volume after tendon release did not differ with or without nandrolone administration; although, fat accumulation was prevented by nandrolone.⁶ This model has been used to replicate chronic tears (e.g., at 16–40 weeks)^{6,21,22} with high intramuscular fat content (around 50%; Goutallier stage 3²³). However, early alterations of muscle volume and composition (i.e., representing an acute tear after 2 weeks) and the impact of nandrolone on the costamere complex have not been investigated yet.

Therefore, the aim of the current study was (i) to define RC muscle volume and composition after tendon release in sheep and (ii) to describe alterations of costamere turnover with and without weekly doses of nandrolone. We specifically hypothesized that muscle remodeling starts early after a RC tear and is already detectable after 2 weeks. Additionally, we hypothesized that changes in muscle volume and composition are accompanied by decreased FAK phosphorylation and increased costamere turnover, assessed by the associated protein levels of FAK, talin, and vinculin. Furthermore, we hypothesized that nandrolone administration not only prevents fat accumulation, but also reduces the loss of contractile tissue and mitigates costamere cleavage.

METHODS

Experimental Design

This experiment was performed according to the Swiss law of animal welfare (TSchG455) and approved by the Veterinary Office of the Canton of Zurich (No. 72/2013). The tendon of the *m. infraspinatus* was surgically released in 18 female Swiss Alpine sheep to simulate a RC tendon tear. Follow-up measurements were performed either 2 weeks (TR2 group; $n=6$; [mean \pm SD] age: 16.6 ± 0.0 months; weight: 59.7 ± 2.5 kg) or 16 weeks after tendon release (TR16 group; $n=6$; age: 23.2 ± 1.2 months; weight: 45.3 ± 4.8 kg). In a third group, sheep underwent the same procedure as in the TR16 group, but also received weekly doses of $150 \mu\text{g}$ nandrolone decanoate in the *m. gluteus maximus* (TR16 + NAN group; $n=6$, age: 23.8 ± 1.2 months; weight: 46.7 ± 2.4 kg). Prior to tendon release (PRE) and 2 (2W) or 16 weeks (16W) after tendon release, alterations in muscle volume were assessed by magnetic resonance imaging (MRI). Furthermore, at 16W, the tendon was repaired in the TR16 and TR16 + NAN groups and measurements were performed again 6 weeks later (REP + 6W) to describe the effects of repair. Biopsy samples were collected from the *m. infraspinatus* to determine the tissue composition and costamere protein levels at the time points: PRE, 2W, 16W, and REP + 6W. The contralateral shoulder served as the control (CC). Sheep were killed after harvesting the last biopsy, which was at 2W for the TR2 group and at REP + 6W for the TR16 and TR16 + NAN groups.

Surgical Tendon Release

Surgery was performed as described previously.⁶ In brief, the tendon of the *m. infraspinatus* was released via osteotomy of the greater tuberosity using an oscillating saw. The tendon and bone chip were wrapped in a silicone tube to prevent spontaneous reattachment. After surgery, sheep were allowed to move freely in the stable.

Single Step Repair

The details of this procedure, which involves removal of the silicon wrap and reattachment of the greater tuberosity to its original site, have been previously described.⁶

Sampling of Muscle Tissue

Approximately 40 mg of tissue (0.02% of average PRE muscle volume) was collected intraoperatively using a Bergstroem needle with a 5 mm diameter (Dixons Surgical Instruments LTD, Wickford, UK). If necessary, samples were rapidly cleaned of blood and immediately frozen in liquid nitrogen cooled isopentane and stored in 2 ml cryotubes at -80°C .

Assessment of Tissue Composition on Biopsy Cross-Section

To assess the tissue composition of the biopsy specimens, consecutive sections (average section area: 6.54 mm^2) were either stained with oil red O to detect fatty tissue (Fig. 1, right), or using the Goldner trichrome technique²⁴ to stain muscle fibers and connective tissue (Fig. 1, left). For the oil red O staining, sections were fixed for 10 min in 4% paraformaldehyde. After washing, sections were incubated for 5 min in 60% isopropanol and then stained for 10 min in the oil red O working solution (40% of 5 g/L oil red O [#0684-100G, VWR, Radnor, PA] in isopropanol; 60% ddH₂O). Then, sections were again incubated in 60% isopropanol, washed, counterstained in hematoxylin, and then rinsed in ddH₂O before coverslips were mounted.

The total area was determined on the oil red O stained section with ImageJ (v1.48v, National Institutes of Health, USA) using the “analyze particles” tool. Afterward, red stained areas (i.e., fatty tissue) were visually isolated, quantified with the same tool, and related to the total area. The area corresponding to contractile tissue was determined from the red-colored muscle fibers on the Goldner stained sections. Then, fatty tissue and contractile tissue were related to the total area. The total area (=100%) – (fatty tissue + contractile tissue) was named “other types of tissue” and covered all extramycocellular tissue apart from fat.

Assessment of Muscle Volume and Composition

To determine muscle volume, MRI scans of both shoulders were performed immediately after surgery with the sheep still under general anesthesia. Scanning and readout analysis were performed as described previously.⁶ The approximate volumes of the different tissue types in the total muscle were calculated by splitting the total volume according to the percentages of fatty, contractile, and other tissue types that were assessed histologically in the biopsy specimen.

Immunoprecipitation of FAK-pY397

Muscle homogenates were prepared from cryosections of muscle biopsies as previously described.²⁵ The protein homogenate ($250 \mu\text{g}$) was brought to a total volume of $750 \mu\text{l}$ with RIPA buffer. This was mixed for 30 min at 4°C under steady rotation (20 rpm) and then centrifuged. Without touching the pellet, $700 \mu\text{l}$ of the homogenate was moved to a new tube and the centrifugation step was repeated. Then, $650 \mu\text{l}$ of the homogenate was moved to a new tube and incubated over night with $200 \mu\text{g}$ protein A-sepharose (#P9424, Sigma-Aldrich, St. Louis, MO) and a combination of anti-pFAK antibodies ($1 \mu\text{l}$ each, #44-624G, Thermo Fisher Scientific, Waltham, MA; #sc-11765-R, Santa Cruz Biotechnology, TX), again under steady rotation at 4°C . The next

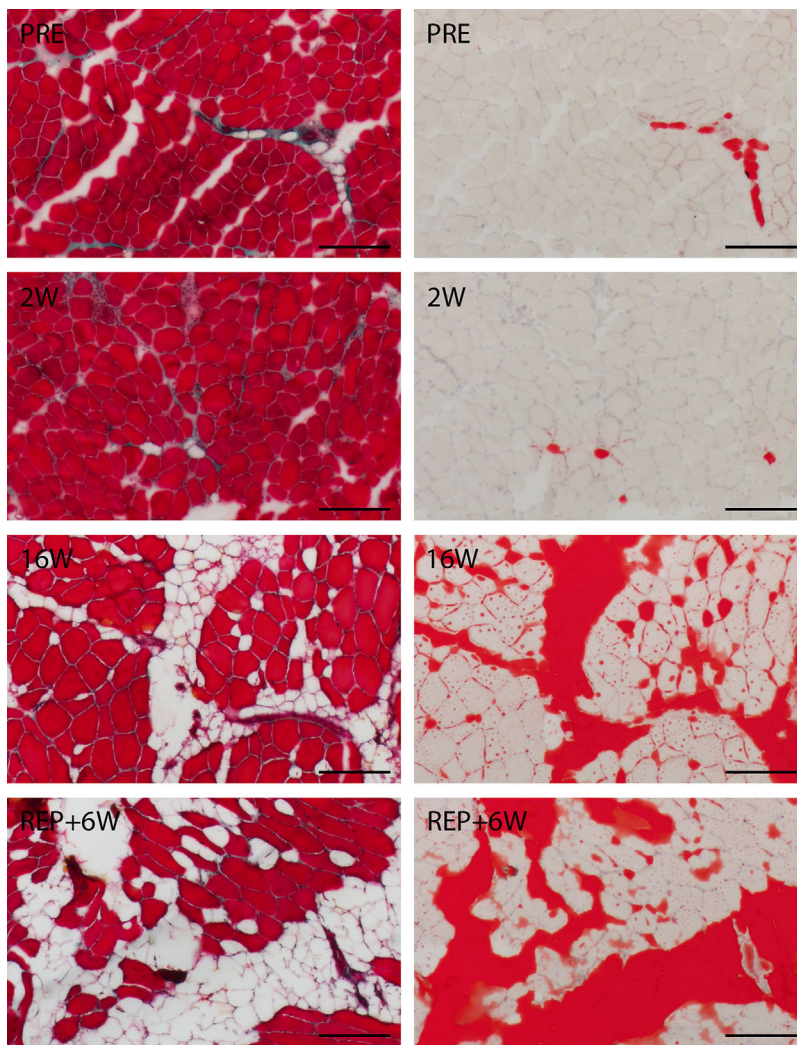


Figure 1. Histologically illustrated temporal changes in tissue composition using representative images of Goldner (left) and oil red O (right) staining at the PRE, 2W, 16W, and REP+6W time points of the groups TR2 and TR16. On Goldner images, contractile tissue is stained red, the membranes of adipocytes are stained pink, and connective tissue is stained pink and green. On oil red O images, fatty tissue is stained red, while contractile and other types of tissue are unstained. Scale bar denotes 200 μ m.

day, the protein A-sepharose was washed twice with RIPA buffer to remove non-bound proteins. The FAK-pY397 was resuspended in 2 \times Laemmli buffer, separated from the immune complex, and stored at -30°C until detection via Western blot.

Assessment of Costamere Protein Levels

Biopsy homogenates were denatured in 2 \times Laemmli sample buffer and separated on 7.5% SDS-PAGE gels (Bio-Rad Mini-protean TGX stain-free), with 10 μ g of protein loaded per lane. To detect FAK-pY397, 10 μ l of precipitate was loaded. Proteins were blotted on a nitrocellulose membrane using the Bio-Rad Trans-Blot Turbo System (Bio-Rad, Cressier, Switzerland). Equal loading and transfer quality were verified using Ponceau S staining. After blocking in 5% milk/1% BSA in TBST, the following antibodies were used to detect the target proteins: FAK-pY397 and total FAK were detected using a home-made polyclonal rabbit α -FAK serum (1:1,000) described previously¹⁷; gamma- and meta-vinculin were detected using a mouse α -vinculin serum (gift of Dr. M. A. Glukhova, Paris, France; 1:100); and talin and integrin-beta6 were detected using the monoclonal mouse α -talin antibody #ab95034 (Abcam, Cambridge, UK; 1:100) and the polyclonal mouse α -ITGB6 antibody #ab169271 (Abcam; 1:500), respectively. Then, the following secondary antibodies (1:20000) were applied on

the respective membranes: Goat α -mouse antibody #A9917 (Sigma-Aldrich) or mouse α -rabbit antibody #55676 (MP Biomedicals, OH). The signals were recorded using the PXi System (Syngene, Labgene Scientific SA, Chatel St Denis, Switzerland) and quantified using the rectangular mode in Quantity One (Bio-Rad) according to the user manual. To remove inter-membrane variability, signals were first divided by a standard loaded on every gel and after that no statistically significant group differences were detected at baseline, values were scaled per group to set the mean of the PRE-values to 1 to display relative changes compared with baseline.

Localization of Costamere Proteins

To localize FAK, myosin heavy chain and FAK were co-stained on tissue sections following the oil red O staining (Fig. 2, right). On another microscopic slide, talin and FAK were co-stained (Fig. 2, left). In brief, sections were fixed for 10 min in 4% paraformaldehyde and blocked in a humid chamber in 5% normal goat serum in phosphate buffer saline and 0.2% triton (PBS-triton) for 1 h at room temperature. Primary antibody incubation was performed either in a mix of mouse α -myosin heavy chain antibodies (α -slow muscle myosin #MAB1628, Millipore Corp., Temecula, CA; α -fast skeletal myosin #M4276, Sigma-Aldrich; both 1:100) and rabbit anti-FAK #sc-557 (Santa Cruz Biotechnology,

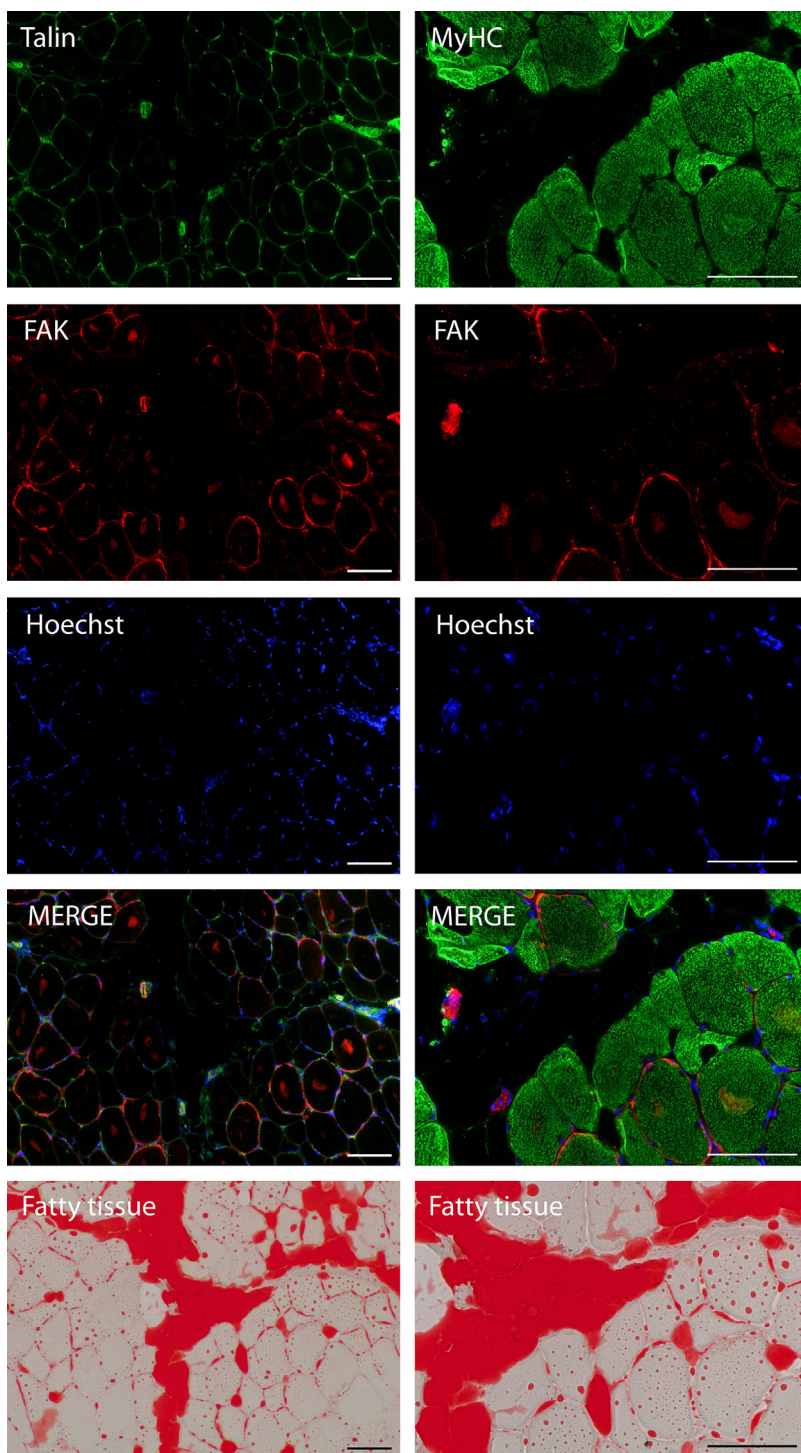


Figure 2. Immunofluorescence images co-stained with talin and FAK (left column) and myosin heavy chain (MyHC) and FAK (right column) followed by consecutive oil red O images (bottom row) of a representative TR16 sheep at 16 W. Scale bar denotes 100 μ m.

TX; 1:50), or in rabbit anti-FAK and mouse anti-talin (#T3287, Sigma-Aldrich; 1:50). After 1 h, incubation was stopped and slides were washed in PBS-triton. Then, sections were incubated in a mix of the secondary antibodies, anti-mouse Alexa Fluor[®] 488 #A11017 and anti-rabbit Alexa Fluor[®] 555 #A21428 (Thermo Fisher Scientific) diluted 1:200 in PBS-triton, for 45 min. After washing with PBS, nuclei were stained with Hoechst #62249 (Thermo Fisher Scientific; 1:2,000) in PBS for 1 min before coverslips were mounted.

Statistical Analysis

Data are presented as the mean \pm SD. To detect differences between time points and groups, a general linear model with repeated measures was applied with “time point” as the within-subjects variable (repeated) and “group” as the between-subjects factor (split-plot ANOVA). A bivariate, two-tailed Pearson’s test revealed correlations between protein levels and muscle composition. For the statistical analysis, SPSS Statistics v22.0 (IBM, Chicago, IL) was used. Statistical significance was defined as $p < 0.05$.

Muscle volume and composition

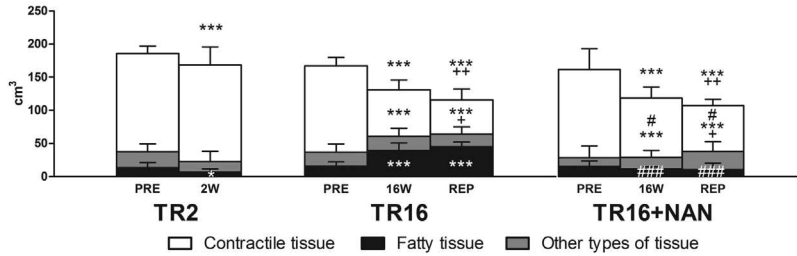


Figure 3. Muscle volume and composition. Indications above columns refer to alterations in total volume, indications inside columns refer to alterations in the volume of the specific tissue type. The volume of other types of tissue was calculated from the total volume (contractile tissue + fatty tissue). * $p < 0.05$, *** $p < 0.001$ for time effect compared with PRE within the same group; $^{\dagger}p < 0.05$, $^{++}p < 0.01$ for time effect compared with 16W within the same group; $^{\#}p < 0.05$, $^{###}p < 0.001$ for group effect compared with TR16 at the same time point. Values are means + SD.

RESULTS

Tendon Release Leads to Reduction of Muscle Volume and Changes in Tissue Composition

The initial muscle volume and composition did not differ between groups (Fig. 3). In TR2 and TR16, tendon release led to a decrease in muscle volume, which was already detectable at 2 W ($-17.0 \pm 5.0 \text{ cm}^3$, $p < 0.001$ compared with PRE) and was more pronounced at 16 W ($-35.8 \pm 12.1 \text{ cm}^3$, $p < 0.001$ compared with PRE). At REP + 6W, muscle volume was further decreased ($-51.2 \pm 15.0 \text{ cm}^3$, $p < 0.001$ compared with PRE; $-15.3 \pm 12.8 \text{ cm}^3$, $p = 0.004$ compared with 16W; Fig. 3). Microscopy images representing the relative distribution of tissue types are shown in Figure 1 and the percent areas from histological analysis that were used to calculate the volumes of contractile, fatty, and remaining tissue are presented in Figure 4.

In TR2 and TR16, the volume of contractile tissue remained unchanged at 2 W ($-2.0 \pm 25.6 \text{ cm}^3$, $p = 0.859$ compared with PRE), but was significantly decreased at 16 W ($-59.4 \pm 10.2 \text{ cm}^3$, $p < 0.001$ compared with PRE). Contractile tissue was further reduced at REP + 6W ($-77.9 \pm 17.9 \text{ cm}^3$, $p < 0.001$ compared with PRE; $-18.6 \pm 16.7 \text{ cm}^3$, $p = 0.028$ compared with 16W; Fig. 3). The volume of fatty tissue was reduced at 2 W ($-6.4 \pm 5.9 \text{ cm}^3$, $p = 0.045$ compared with PRE) but then increased at 16W ($+23.6 \pm 12.8 \text{ cm}^3$, $p < 0.001$ compared with PRE) in TR2 and TR16, respectively. At REP + 6W ($+29.4 \pm 11.6 \text{ cm}^3$, $p < 0.001$ compared with PRE), it was similar to 16W ($+5.8 \pm 11.1 \text{ cm}^3$, $p = 0.186$; Fig. 3). Fatty tissue measured in the biopsy sample correlated quantitatively with the fat portion ($r = 0.756$, $p < 0.001$, $N = 66$) assessed using the Dixon method (described by Gerber et al.)⁶ on the MRI scans (data not shown). Tendon release did not influence the volume of other tissues (i.e., non-myocellular and non-fatty tissue; Fig. 3).

Nandrolone Does Not Influence Total Muscle Volume, but Reduces the Loss of Contractile Tissue and Prevents Fat Accumulation

In TR16 + NAN, the total muscle volume was decreased at 16 W ($-43.5 \pm 18.3 \text{ cm}^3$, $p < 0.001$ compared with PRE). At REP + 6 W, muscle volume was further decreased ($-54.7 \pm 19.0 \text{ cm}^3$, $p < 0.001$ compared with PRE; $-11.2 \pm 5.8 \text{ cm}^3$, $p = 0.020$ compared with 16W; Fig. 3). In the TR16 + NAN group, the volume of contractile tissue was reduced at 16 W ($-43.7 \pm 22.1 \text{ cm}^3$, $p < 0.001$ compared with PRE) and at REP + 6 W ($-64.3 \pm 35.0 \text{ cm}^3$, $p < 0.001$ compared with PRE; $-20.5 \pm 18.7 \text{ cm}^3$, $p = 0.018$ compared with 16W). Compared with TR16, the volume of contractile tissue in TR16 + NAN was significantly larger at 16 W ($+20.7 \text{ cm}^3$, $p = 0.046$) and REP + 6 W ($+18.8 \text{ cm}^3$, $p = 0.036$; Fig. 3). The volume of fatty tissue remained unchanged in the nandrolone-treated sheep at both 16 W ($-4.0 \pm 7.0 \text{ cm}^3$, $p = 0.368$) and REP + 6 W ($-4.6 \pm 8.0 \text{ cm}^3$, $p = 0.283$) compared with PRE. This corresponds to a significantly lower volume of fatty tissue compared with TR16 at both 16 W (-27.8 cm^3 , $p < 0.001$) and REP + 6 W (-34.3 cm^3 , $p < 0.001$; Fig. 3). Nandrolone did not influence the volumes of other types of tissue (Fig. 3).

Tendon Release Leads to a Relative Deactivation of FAK on the Y397 Site, Which Is Prevented by Nandrolone Administration

Representative Western blot images are shown in Figure 5. The majority of FAK protein is localized around muscle fibers and not fatty tissue (Fig. 2). Its relative activity, expressed as the ratio of Y397-phosphorylated FAK per total FAK, remained unchanged after 2 weeks of tendon release ($-8.2 \pm 41.0\%$, $p = 0.545$ compared with PRE), but was significantly reduced at 16 W ($-27.1 \pm 20.6\%$, $p = 0.039$ compared

Relative tissue distribution

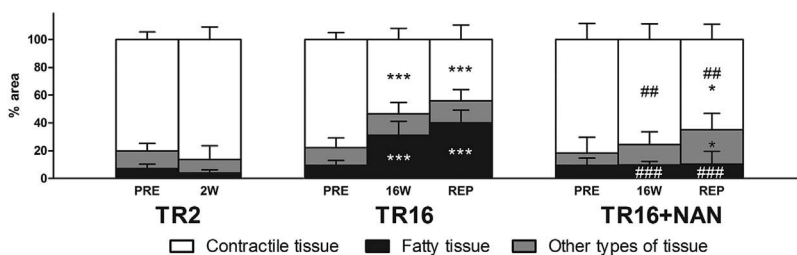


Figure 4. Relative tissue distribution assessed on biopsy cross-sections. Indications inside columns refer to alterations in the % area of the specific tissue type. The % of other types of tissue was defined as 100% (contractile tissue + fatty tissue). * $p < 0.05$, *** $p < 0.001$ for time effect compared with PRE within the same group; $^{\#}p < 0.01$, $^{###}p < 0.001$ for group effect compared with TR16 at the same time point. Values are means + SD.

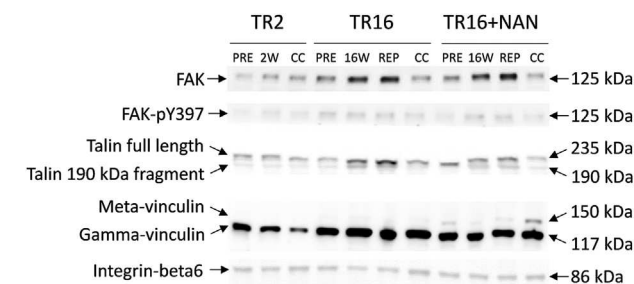


Figure 5. Representative Western blot images of focal adhesion kinase (FAK), its phosphorylated form FAK-pY397, full length talin and its 190 kDa fragment, gamma- and meta-vinculin, and integrin-beta6. Relative protein amounts were assessed at baseline (PRE), 2 weeks (2W), or 16 weeks (16W) after tendon release and 6 weeks after repair (REP + 6W). The measurements in the contralateral control (CC) took place at the end of the intervention.

with PRE). At REP + 6W, the initial level of relative FAK activity was not reestablished ($-34.2 \pm 19.5\%$, $p = 0.010$ compared with PRE; Fig. 6C). The total amount of FAK protein was unchanged at 2W but tended to increase after 16 weeks of tendon release ($+44.6 \pm 33.5\%$, $p = 0.062$ compared with PRE), and was only significantly increased after repair compared with baseline ($+94.6 \pm 61.5\%$, $p = 0.012$; Fig. 6A). Nandrolone prevented the deactivation of FAK on the Y397 site, after 16 weeks of tendon release, and after the subsequent 6 weeks of repair (Fig. 6C), as the total amount of FAK (Fig. 6A) remained unchanged.

Tendon Release and Subsequent Repair Leads to Increased Costamere Turnover

Representative Western blot images are shown in Figure 5. Tendon release did not exert an effect on talin quantity, but 6 weeks of repair significantly increased it ($+241.4 \pm 160.4\%$, $p < 0.001$ compared

with PRE; Fig. 7A). Instead, the 190 kDa fragment of talin was increased already at 16W ($+122.4 \pm 60.3\%$, $p = 0.003$) and remained elevated at REP + 6W ($+161.1 \pm 84.4\%$, $p = 0.026$ compared with PRE; Fig. 7B). Correlation analysis revealed that the abundance of the 190 kDa fragment of talin is tightly associated with the levels of FAK ($r = 0.801$, $p < 0.001$; Fig. 8). Cleavage of talin was also associated with the loss of contractile tissue ($r = -0.407$, $p < 0.001$; Fig. 8). Similar to full length talin, gamma-vinculin was not affected by tendon release, but was increased with subsequent repair ($+105.9 \pm 111.6\%$, $p = 0.017$ compared with PRE; Fig. 7C). Meta-vinculin remained unaffected by tendon release and repair (Fig. 7D). The integrin-beta6 protein level dropped after 2 weeks of tendon release ($-44.6 \pm 36.0\%$, $p = 0.029$) and the resting level was reestablished at 16W. Repair did not influence integrin-beta6 protein expression (Fig. 7E).

Nandrolone Prevents Changes in Costamere Turnover After Tendon Release

In the TR16 + NAN group, tendon release had no effect on the relative quantities of talin, its 190 kDa fragment, gamma- and meta-vinculin, and integrin-beta6 (Fig. 7A–E). Instead, 6 weeks after repair, the amount of full length talin protein was significantly decreased compared with TR16 (-65.0% , $p = 0.031$; Fig. 7A). Concomitantly, the 190 kDa fragment of talin was increased at REP + 6W compared with baseline ($+175.7 \pm 195.9\%$, $p = 0.017$; Fig. 7B). Compared with TR16, nandrolone kept the gamma-vinculin protein level similar to baseline after repair (Fig. 7C). Furthermore, after repair, meta-vinculin and integrin-beta6 quantities were not distinguishable from baseline or the respective level in the TR16 group (Fig. 7D and E).

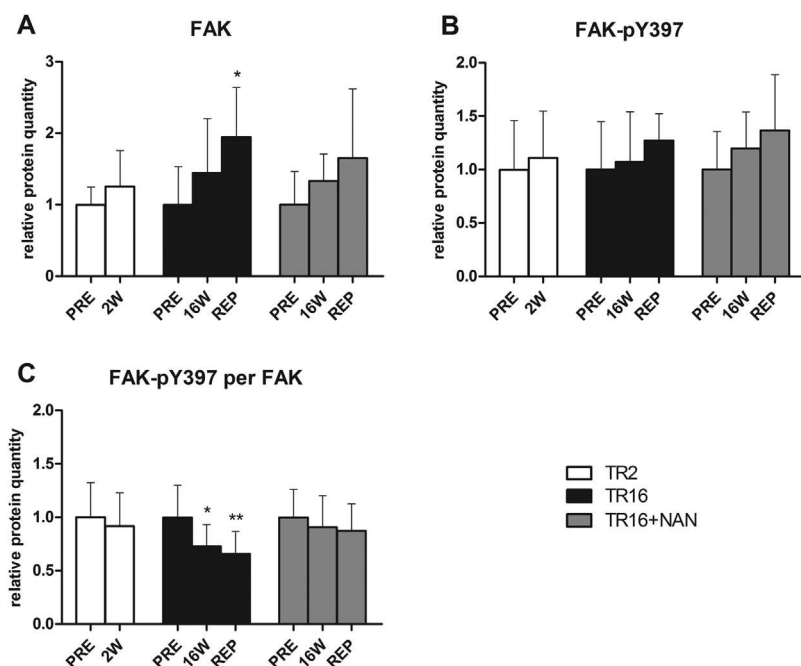


Figure 6. Relative protein quantities of (A) total FAK, (B) FAK-pY397, and (C) the amount of FAK-pY397 per total FAK. * $p < 0.05$, ** $p < 0.01$ for time effect compared with PRE within the same group. Values are means + SD.

DISCUSSION

The purpose of this study was to define the RC muscle composition after tendon release in sheep and to describe costamere turnover with and without weekly doses of nandrolone. In this model, fat accumulated and costamere turnover increased at 16 weeks, but not 2 weeks after tendon release and these effects were prevented by nandrolone. Furthermore, nandrolone mitigated the loss of contractile tissue.

Assessments of total muscle volumes via MRI revealed that muscle remodeling starts immediately after tendon release, as the total muscle volume was already reduced at 2 weeks (Fig. 3). This loss in volume continued until 16 weeks and was not stopped by repair (Fig. 3). The relative loss of muscle volume corresponds well to the magnitude and time course of atrophy observed in humans during bed rest, unilateral limb suspension, immobilization, and spaceflight studies,²⁶ indicating that the ovine model might also be suitable for replication of human muscle unloading situations other than the RC tear. Interestingly, the reduction in muscle volume after 2 weeks is, in part, explained by a decreased volume

of fat but not contractile tissue (Fig. 3). Liu et al.²⁷ showed previously, in a rat model, that fat accumulation is not detectable at 2 weeks after RC tendon release; while, it is present after 6 weeks. Also in sheep, fat accumulation was detectable on MRI scans after 6 weeks,⁶ indicating the turning point for fat accumulation is between 2 and 6 weeks after tendon release. As blood flow, and thereby delivery of substrates, is contraction dependent,²⁸ we hypothesize that the unloaded muscle has to live on its local energy stores (i.e., intramuscular fatty tissue), leading to decreased fat content until pathological fat accumulation occurs. The findings that the contractile tissue volume remained unchanged after 2 weeks and was significantly decreased after 16 weeks (Fig. 3) support the conclusion that pathological muscle loss does not start before 2 weeks after the injury.

The present study revealed that nandrolone protects contractile tissue. The mitigated loss of contractile tissue is the reason for similar total muscle volumes with/without nandrolone despite significantly lower fat content in the nandrolone-treated sheep

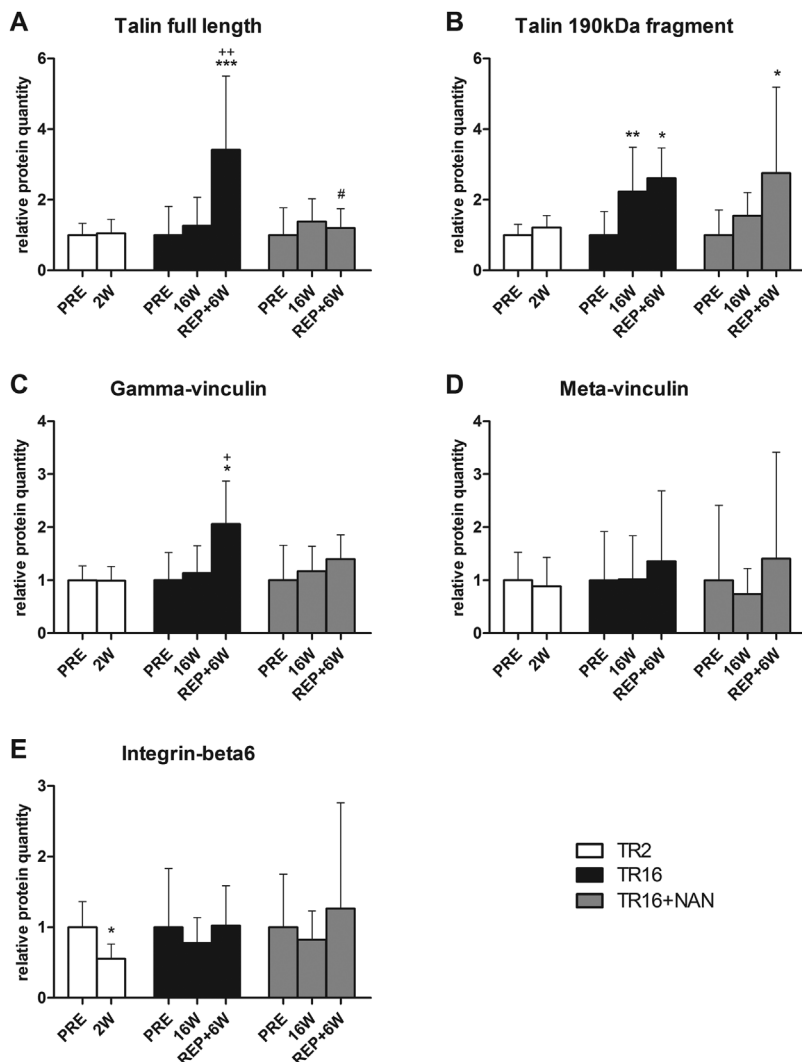


Figure 7. Relative quantities of the costamere proteins. (A) Full length talin, (B) the proteolytic 190 kDa fragment of talin, (C) gamma-vinculin, (D) meta-vinculin, and (E) integrin-beta6. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ for time effect compared with PRE within the same group; + $p < 0.05$, ++ $p < 0.01$ for time effect compared with 16W within the same group; # $p < 0.05$ for group effect compared with TR16 at the same time point. Values are means + SD.

Pearson correlations		Muscle volume	Contractile tissue	Fatty tissue	Other types of tissue	FAK	FAK-pY397	FAK-pY397 per total FAK	Talin full length	Talin 190kDa fragment	Gamma-vinculin	Meta-vinculin	Integrin-beta6
Muscle volume	<i>r</i>		.880	-.321	.001	-.267	-.283	.064	-.207	-.321	-.236	.075	.148
	<i>p</i>		.000	.009	.991	.030	.021	.609	.096	.009	.057	.547	.235
Contractile tissue	<i>r</i>	.880		-.521	-.347	-.355	-.264	.169	-.303	-.407	-.286	.008	.080
	<i>p</i>	.000		.000	.004	.003	.032	.176	.013	.001	.020	.947	.523
Fatty tissue	<i>r</i>	-.321	-.521		-.178	.175	-.036	-.277	.325	.235	.141	-.021	-.110
	<i>p</i>	.009	.000		.153	.160	.776	.024	.008	.058	.258	.866	.378
Other types of tissue	<i>r</i>	.001	-.347	-.178		.218	.143	-.057	.076	.193	.136	.139	.176
	<i>p</i>	.991	.004	.153		.079	.250	.651	.543	.121	.276	.265	.157
FAK	<i>r</i>	-.267	-.355	.175	.218		.616	-.588	.638	.801	.605	.429	.624
	<i>p</i>	.030	.003	.160	.079		.000	.000	.000	.000	.000	.000	.000
FAK-pY397	<i>r</i>	-.283	-.264	-.036	.143	.616		.173	.298	.479	.452	.228	.472
	<i>p</i>	.021	.032	.776	.250	.000		.166	.015	.000	.000	.066	.000
FAK-pY397 per total FAK	<i>r</i>	.064	.169	-.277	-.057	-.588	.173		-.418	-.452	-.372	-.239	-.285
	<i>p</i>	.609	.176	.024	.651	.000	.166		.000	.000	.002	.053	.021
Talin full length	<i>r</i>	-.207	-.303	.325	.076	.638	.298	-.418		.503	.581	.341	.463
	<i>p</i>	.096	.013	.008	.543	.000	.015	.000		.000	.000	.005	.000
Talin 190kDa fragment	<i>r</i>	-.321	-.407	.235	.193	.801	.479	-.452	.503		.404	.529	.623
	<i>p</i>	.009	.001	.058	.121	.000	.000	.000	.000		.001	.000	.000
Gamma-vinculin	<i>r</i>	-.236	-.286	.141	.136	.605	.452	-.372	.581	.404		.225	.414
	<i>p</i>	.057	.020	.258	.276	.000	.000	.002	.000	.001		.070	.001
Meta-vinculin	<i>r</i>	.075	.008	-.021	.139	.429	.228	-.239	.341	.529	.225		.585
	<i>p</i>	.547	.947	.866	.265	.000	.066	.053	.005	.000	.070		.000
Integrin-beta6	<i>r</i>	.148	.080	-.110	.176	.624	.472	-.285	.463	.623	.414	.585	
	<i>p</i>	.235	.523	.378	.157	.000	.000	.021	.000	.000	.001	.000	

<i>r</i>	≥ 0.8	≥ 0.6	≥ 0.4	≥ 0.2	≤ -0.2	≤ -0.4	≤ -0.6	≤ -0.8
<i>p</i> < 0.05								

Figure 8. Pearson correlations for all groups (TR2, TR16, TR16 + NAN) and all time points (PRE, 2W, 16W, REP + 6W, CC). Results are presented as the correlation coefficient *r* and *p*-value; *N* = 66.

(Fig. 3). It was shown previously that nandrolone reduces unloading-induced loss of muscle protein content in mice.¹⁹ The present study confirmed this also occurs with RC tendon release in sheep. Furthermore, exclusive assessment of the relative tissue distribution does not sufficiently describe shifts in tissue types if the total volume, which the distribution is related to, varies throughout the intervention, for example, contractile tissue in the TR16 + NAN group at 16W was lost without a relative change in tissue

distribution (compare Fig. 3 with Fig. 4). Conversely, the assessment of total muscle volume alone does not allow conclusions to be drawn about its composition (Fig. 3).

The breakdown of contractile tissue has to be coupled to the disassembly of its structural anchors.¹⁰ The costamere is such an anchor and the mechanosensor, FAK, its main regulator, is very sensible to alterations of muscle loading.¹⁵ The relative decrease of FAK-pY397 (Fig. 6) reflects

reduced loading, which was previously shown after unloading of human *m. vastus lateralis*.¹⁶ Similar to our results, total FAK protein tended to increase in a human model with unloading by limb suspension¹⁶ and bedrest.²⁹ Apart from FAK, one of the main structural costamere components, the protein talin, was also affected by tendon release. The increase of the 190 kDa talin fragment at 16 W (Fig. 7) may be indicative of increased costamere turnover, as the rise in the fragment did not decrease the amount of full length protein; thus, the full length talin seems to be replaced immediately. This 190 kDa fragment of talin originates from cleavage of the full length protein by the Ca^{2+} -activated protease calpain.³⁰ The calpain system is responsible for initial cleavage of structural proteins like the costamere to release sarcomeres and make them accessible for quantitative degradation by the proteasome system.¹⁰ Indeed, the increased cleavage of talin was associated with loss of contractile tissue (Fig. 8). There were no changes in costamere turnover (Figs. 6C and 7A and B) or loss of contractile tissue (Fig. 3) at 2 weeks; while, both were present at 16 weeks after tendon release. Nandrolone treatment prevented both the decrease of relative FAK activation and talin cleavage by calpain, probably by lowering Ca^{2+} -levels¹⁹ and thereby decreasing calpain activity.^{10,12} In general, costamere protein levels seem to be predictive of contractile tissue volume (and vice versa; Fig. 8), but further study is needed to determine whether there is a causal connection between preservation of normal costamere turnover and mitigated loss of contractile tissue. Interestingly, repair increased the relative amounts of FAK, talin, and gamma-vinculin in the TR16 group, indicating that the number of costameres increased, as talin and gamma-vinculin are some of the main structural components of the costamere¹¹ and FAK acts as a main regulator.¹⁷

This study has several limitations. First, the muscle biopsy was harvested from one site only and does not necessarily represent the entire muscle; although, fatty tissue measured in the biopsy correlated quantitatively with the fat portion measured on MRI scans. Furthermore, the sample was harvested from the distal third of the lateral portion of the muscle, which is close to the myotendinous junction. This area was previously described as having the highest degree of fat accumulation in rat³¹ and rabbit^{32,33} models of RC tears. Due to potential regional differences, the calculated volumes of contractile, fatty, and other types of tissue should be considered as approximate values. Furthermore, the nandrolone-untreated sheep had to be divided into two groups, which was necessary because local authorities declared the surgical interventions as too numerous/frequent to be ethical for one group alone. Nevertheless, statistical analysis did not identify group differences in any PRE muscle characteristics.

CONCLUSIONS

In the ovine model of RC tears, muscle atrophy starts with the tendon tear and takes place in two stages. In the first 2 weeks, atrophy is associated with reduced fat content and preservation of contractile tissue. Thereafter, there is substantial fat accumulation and loss of contractile tissue. Degradation of contractile tissue is tightly associated with increased costamere turnover and can be mitigated by the administration of nandrolone decanoate.

OUTLOOK

Future studies should address whether the repair of acute tears is possible and successful if started within 2 weeks after the tear, as contractile tissue is not quantitatively lost by this time point, at least in this animal model. The finding that intramuscular fat decreases before it accumulates leads to questions about the timing of this turning point and what causes it. Moreover, the functional relevance of costamere protein cleavage on the degradation of contractile tissue needs to be determined. In addition, the clinical benefit of early administration of nandrolone decanoate needs to be further elaborated.

AUTHORS' CONTRIBUTIONS

Conception and design of research: MF, CG; performed animal experiments: KW, DCM, BVR, MCB; performed muscle analysis: SR, CBM; analyzed data: SR, CBM, KW; interpreted the results of experiments: SR, MF, CG; funding: MF, CG; prepared figures: SR; drafted the manuscript: SR; edited and revised the manuscript: SR, MF, CG, BVR; and approved the final version of the manuscript: All authors.

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